REMARKS

Restriction Requirement

The Examiner has required the Applicant to elect one of the following inventions for further examination: Group I, Claims 1 – 26 and 41, drawn to a chemical composition comprising a ß-strand-forming peptide having characteristics that (i) said peptide comprises a sequence of at least four consecutive amino acid residues that are ß-strand formers, and (ii) said peptide comprises amino acids whose side chains undergo Nα-modification; Group II, Claims 27 – 30, 34 – 36 and 44, drawn to a method of inhibiting aggregation of target β-strands, or aggregation of monomer proteins or aggregation of protein subunits or enzyme, comprising contacting the Group I composition with said β-strand, monomer proteins or protein subunits; Group III, Claims 31- 32, drawn to a method of assisting in refolding denatured or aggregated protein comprising contacting the denatured or aggregated protein with composition of Group I; Group IV, Claims 33, 42 – 43, drawn to a method of diagnosing or treating disease associated with protein aggregation comprising contacting the aggregated protein with the Group I composition; Group V, Claims 37 – 38, drawn to a method of indicating the presence of β-structure(s) comprising exposing a test sample to a composition comprising the composition of Group I that has a detectable moiety; Group VI, Claim 39, drawn to a method of performing a protein denaturation chromatography comprising covalently attaching the Group I composition to a solid matrix or resin, running a test sample through chromatographyic column, and separating desired product from said sample; Group VII, Claim 40, drawn to a combinational library comprising the Group I composition.

Pursuant to the restriction requirement, Applicant elects the invention of Group I, Claims 1 – 26 and 41, drawn to a chemical composition comprising a ß-strand-forming peptide having characteristics that (i) said peptide comprises a sequence of at least four consecutive amino acid residues that are ß-strand formers, and (ii) said peptide comprises amino acids whose side chains undergo Nα-modification, for further examination, **with traverse**.

The Examiner has indicated that the invention listed as Groups I – VII do not related to a single general invention concept under PCT Rule 13.1 because they lack the same or corresponding technical features. More specifically, the instant Claim 1 has been allegedly deemed obvious over Quibell M. et al. (J. Chem. Soc. Perkin. Trans (1995) 1, 2019 – 2024), hereinafter Quibell.

Applicant traverses the restriction requirement and respectfully submits that Quibell reference does not render Claim 1 obvious. The substituents disclosed in Quibell are designed as synthetic <u>intermediates</u> to prevent aggregation of the β-amyloid molecules, with themselves, during their synthesis. The sole purpose of the Nα-substituents in the molecules of the Quibell reference is to prevent <u>any</u> association whatsoever with other identical molecules, so that they remain soluble and monomeric during synthesis. The strategy Quibell uses to prevent aggregation is to position AcHmb substituent groups on <u>both</u> sides (edges) of the β-amyloid to prevent the formation of hydrogen bonds between separate β-amyloid molecules.

Even when the molecules of Quibell do not function as expected (as discussed in the second paragraph, column 2 of page 2019), they are still unable to associate along <u>only one</u> edge. Instead, they associated along both edges (through inter- and intramolecular associations), as shown in Figure 1(b) of the Quibell document. This is because the β-strand-forming section of the Quibell peptide does not comprise "at least one" Nα-substituent, as the embodiments of the present invention indicate.

Furthermore, the present invention relates to the formation of synthetic compounds designed to prevent the aggregation of naturally occurring β-amyloids. The <u>α-D-amino acids</u> used in the compounds of the present invention are non-natural, whereas the β-amyloids of the Qubell reference are synthesized from naturally occurring <u>α-L-amino acids</u>. Moreover, the L- and D-amino acids are anantiomers with different biological activities, as shown in the enclosed paper by Robert J. Chalifour et al (See Robert J. Chalifour et al., Stereoselective Interactions of Peptide Inhibitors with the β-

Amyloid Peptide, The Journal of Biological Chemistry, (2003), vol. 278, No. 37 pp. 34874 – 34881). Therefore, the compounds of Quibell reference differ from those embodiments of the present invention not only by the positioning of substituents, but also by the amino acids they comprise.

Finally, Applicant respectfully enclosed herewith the copy of Section 33.35 of the PCT Handbook, which states that PCT Contracting States have agreed to follow the unity of invention practice under the PCT. Since no unity of invention objection was raised by the ISA or IPEA with regards to the present invention, Applicant respectfully submits that a designated office "ought not" to raise a lack of unity of invention objection (See PCT Handbook Rel 6 (2000), Sec. 33.35).

In light of the above comments, Applicant respectfully submits that the compounds covered by Claim 1 of the present invention are not obvious over Quibell. Therefore, Groups I to VII are linked by a special technical feature and the restriction requirement is improper.

An action on the merits of all of the claims and a Notice of Allowance thereof are respectfully requested.

Respectfully submitted,

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Enclosures:

(1) Robert J. Chalifour et al., Stereoselective Interactions of Peptide Inhibitors with the β-Amyloid Peptide, The Journal of Biological Chemistry, (2003), vol. 278, No. 37 pp. 34874 – 34881
(2) PCT Handbook Rel 6 (2000), Sec. 33.35

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Stereoselective Interactions of Peptide Inhibitors with the β-Amyloid Peptide*

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Residues 16–20 of the β-amyloid peptide (Aβ) function as a self-recognition element during Aβ assembly into fibers. Peptides containing this motif retain the ability to interact with Aβ and, in some cases, potently inhibit its assembly. Replacing L- with D-amino acids could stabilize such peptides and permit their evaluation as therapeutic agents for Alzheimer's disease. Here we have assessed the effect that such a chiral reversal has on inhibitory potency. D-enantiomers of five peptides, KLVFFA, KKLVFFA, KFVFFA, KIVFFA, and KVVFFA, were unexpectedly more active as inhibitors in an *in vitro* fibrillogenesis assay. Circular dichroism showed that D-KLVFFA more effectively prevented Aβ adopting the β-sheet secondary structure correlated with fibrillogenesis. Electron microscopy showed that fiber formation was also more strongly inhibited by D-KLVFFA. Heterochiral inhibition was confirmed using D-Aβ, on the principle that enantiomeric proteins exhibit reciprocal chiral biochemical interactions. With D-Aβ, L-KLVFFA was the more potent inhibitor, rather than D-KLVFFA. Most significantly, D-peptides were more potent at reducing the toxicity of both $A\beta_{1-40}$ and $A\beta_{1-42}$ toward neuronal cells in culture. This unforeseen heterochiral stereoselectivity of Aβ for D-peptide inhibitors should be considered during future design of peptide-based inhibitors of Aβ neurotoxicity and fibrillogenesis.

β-Amyloid peptide (Aβ)[1] is a 4-kDa peptide that when assembled into amyloid, progressively accumulates in Alzheimer's disease (AD) (1, 2). The neurotoxic properties specifically associated with aggregated forms of this peptide provide key causal evidence linking Aβ to the pathology of AD (3, 4). This link is supported by Aβ immunization experiments in transgenic mice expressing mutant alleles of human amyloid precursor protein. Such vaccination reduces both the Aβ peptide levels and deposits in the brain (5) and also attenuates the associated cognitive impairment in these mice (6, 7). Subsequent studies showed that full assembly of Aβ into mature fibers similar to those found in plaques is not necessary for toxicity. Smaller aggregates with molecular weights corresponding to dimers (8, 9), trimers, and tetramers (10) exhibit toxicity in cell culture as well. These findings support and highlight the necessity that Aβ assembly intermediates be included as targets in AD drug development.

While the delineation of which assembly intermediate(s) retain toxicity will aid in the rational design of specific inhibitors, screening assays have already identified a number of antifibrillogenic compounds (11–13). Studies show that the $A\beta_{16-20}$ region is important for Aβ assembly (14, 15), possibly as an Aβ self-recognition motif (16). These findings led to the development of a series of peptides incorporating this sequence, which bind to the homologous region of Aβ and block its oligomerization (17). In another study an eleven residue peptide containing three prolines, which have a low propensity to form β-sheets, inhibited Aβ fibril formation (18). Peptides that protect against Aβ neurotoxicity in cell culture have been prepared by combining the Aβ-recognition motif with a disruptive element comprised of charged amino acids (19). Other inhibitors of Aβ assembly were developed by modifying peptides containing the Aβ-recognition motif with cholyl groups (20) or with *N*-methyl amino acids (21).

The usefulness of this family of peptidic antifibrillogenic agents may depend on reducing their inactivation by proteolysis. One approach to do this is the replacement of L- with D-amino acids, which creates peptide bonds that are resistant to proteases. This approach was evaluated with proline-containing inhibitor peptides and was found to improve the inhibitor stability without altering the antifibrillogenic activity (18). Similarly a cholyl-modified peptide exhibited activity when prepared with D-amino acids (20). It is fortuitous that the mirror image conformational change in these peptides, in going from L- to D-forms, did not have a profound negative effect on their potency. This might be expected given the effects of stereochemistry on Aβ assembly (22) and other protein/protein interactions (23).

In the present study we investigated this question by quantitatively evaluating the effect of L- to D-amino acid substitutions, on the potency of peptide inhibitors of Aβ assembly. We assessed the antifibrillogenic activity using Thioflavin T fluorescence, circular dichroism and electron microscopy. Unexpectedly, we found that the D-enantiomers of these peptides were considerably more potent at inhibiting Aβ fibrillogenesis than the original L-peptides. To confirm this counterintuitive finding, we showed that fibrillogenesis of the enantiomer of Aβ, D-Aβ, was conversely more potently inhibited by an L-enantiomer inhibitor peptide. Most significantly, the D-enantiomers more efficiently blocked Aβ-induced neurotoxicity in neuronal cell cultures. The design and optimization of antifibrillogenic

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[1] The abbreviations used are: Aβ, β-amyloid peptide; AD, Alzheimer's disease; CD, circular dichroism; C of c, coefficient of confidence; EM, electron microscopy; HFIP, 1, 1, 1, 3, 3, 3-hexafluoroisopropanol; ThT, thioflavin T; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WST-1, (4-[3-(iodophenyl)-2-(4-nitrophenyl)-2,4-5 tetrazolio]-1,3-benzene disulfonate).

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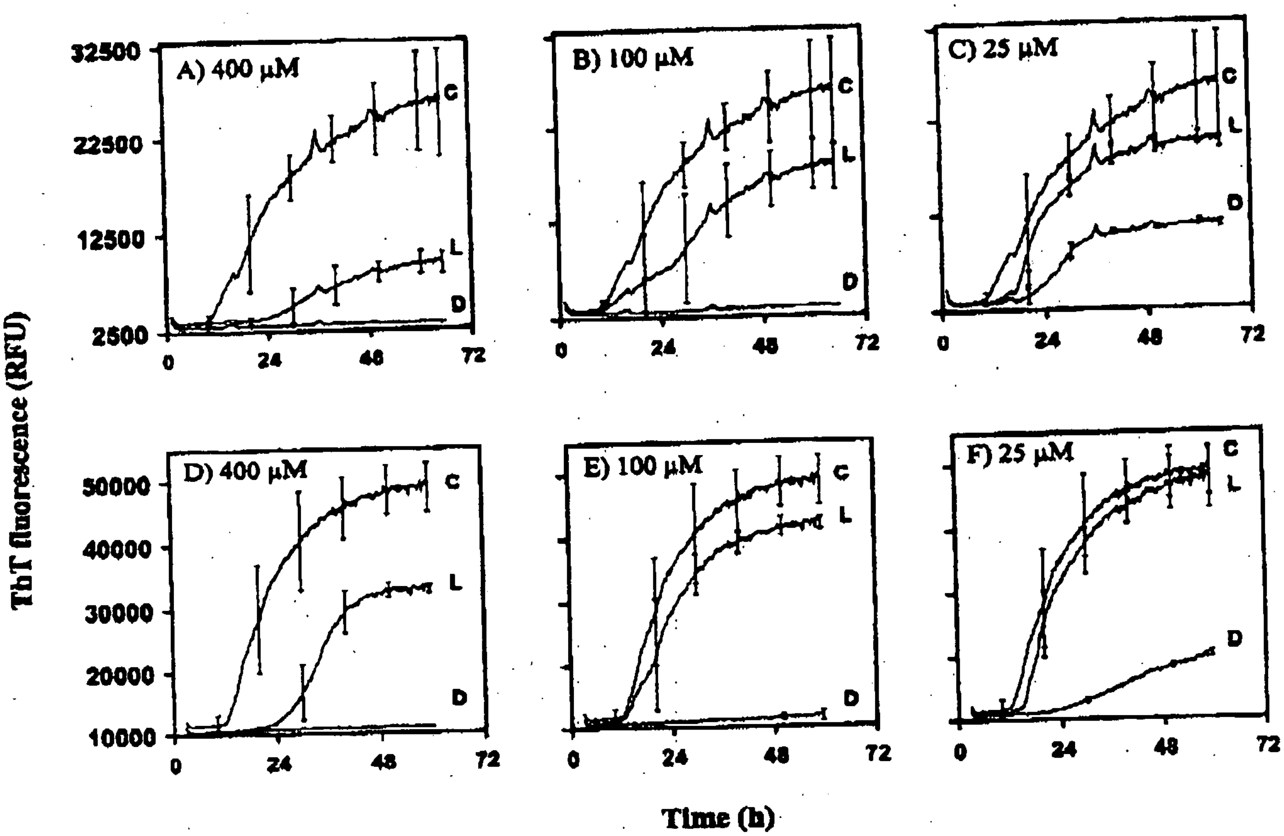

FIG. 1. **Inhibition of $A\beta_{1-40}$ fibrillogenesis by D- and L-enantiomers of KLVFFA and KKLVFFA.** Peptide inhibitors and 20 μM $A\beta_{1-40}$ were incubated as described under "Experimental Procedures" for the ThT fibrillogenesis assay. *A*, 400 μM of D-KLVFFA or L-KLVFFA; *B*, 100 μM of D-KLVFFA or L-KLVFFA; *C*, 25 μM of D-KLVFFA or L-KLVFFA; *D*, 400 μM of D-KKLVFFA or L-KKLVFFA; *E*, 100 μM of D-KKLVFFA or L-KKLVFFA; *F*, 25 μM of D-KKLVFFA or L-KKLVFFA. Amyloid formation was quantified with ThT fluorescence. Abbreviations: *C*, no inhibitor; *L*, L-inhibitor; *D*, D-inhibitor. The average of three samples is shown ± S.D.

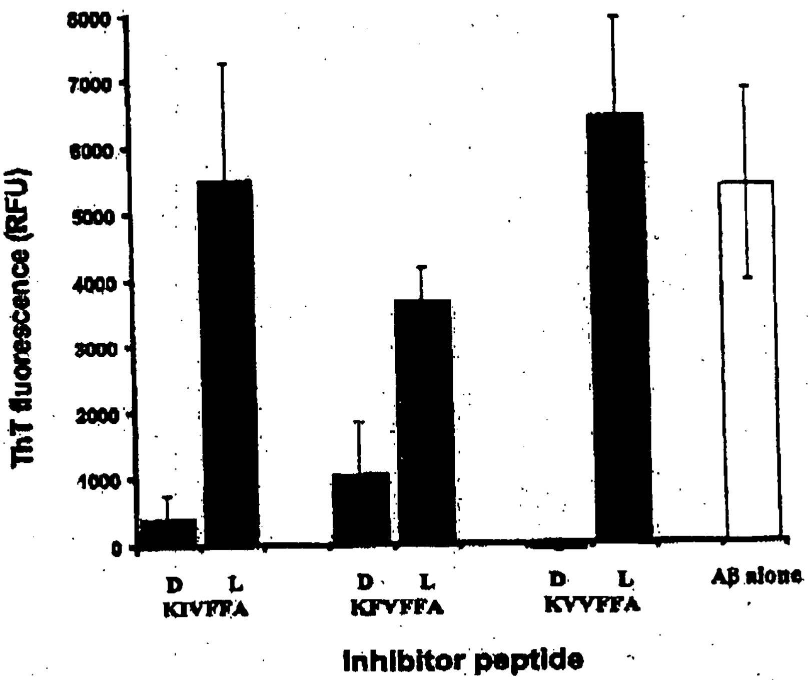

FIG. 2. **Comparison of inhibitor activity of three enantiomeric peptide pairs.** Three enantiomeric peptide pairs were prepared by replacing the leucine of KLVFFA with isoleucine, phenylalanine, or valine. $A\beta_{1-40}$, 20 μM, was incubated as described under "Experimental Procedures" under ThT fibrillogenesis assay, in the presence of 20 μM KIVFFA or KFVFFA enantiomers, or 100 μM of KVVFFA enantiomers. The ThT fluorescence after 10 h of incubation is plotted. The fluorescence of Aβ incubated without inhibitor is shown on the *right*.

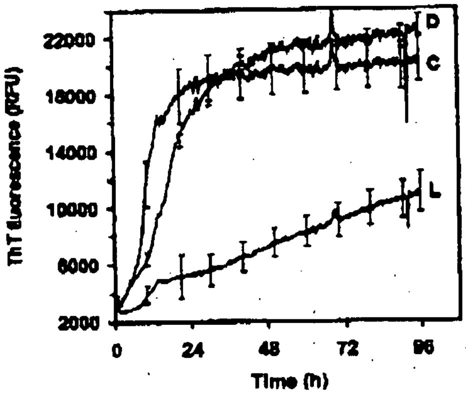

Fig. 3. Reciprocal sensitivity of D-Aβ_{1-40} assembly to peptide inhibitor chirality. D-Aβ_{1-40}, 20 μM, was incubated as described under "Experimental Procedures" for the ThT fibrillogenesis assay, either alone (*C*) or in the presence of either 100 μM L-KLVFFA (*L*) or 100 μM D-KLVFFA (*D*). The average of triplicate incubations is shown ± S.D.

molecules employing the Aβ_{16-21} recognition motif should therefore take into consideration that its interaction with Aβ displays a heterochiral stereoselectivity.

EXPERIMENTAL PROCEDURES

Synthetic Peptides—Aβ_{1-40} and Aβ_{1-42} were prepared by American Peptide Company, Inc., D-Aβ_{1-40} was prepared by Biosource International, Inc. and peptide inhibitors were prepared by ResGen Invitrogen Corp. Peptides were of ≥95% purity. Peptide inhibitors, with the exception of KKLVFFA, were amidated at the C terminus.

Disaggregation of Aβ—Aβ_{1-40} and Aβ_{1-42} were treated to remove aggregates using a modified version of a previously published procedure (24). A 2.5 mg/ml solution of Aβ_{1-40} or Aβ_{1-42} in HFIP (Aldrich) was degassed in a round bottom flask with nitrogen then sonicated in a bath sonicator (VWR) for 1 h. The solution was then filtered through an Anotop 25 Plus 20-nm filter (Whatman). Aliquots were stored in polypropylene tubes at −80 °C until use.

Thioflavin T Fibrillogenesis Assay—An aliquot of Aβ_{1-40} in HFIP was dried under nitrogen in a polypropylene tube. The Aβ_{1-40} was dissolved to 80 μM in 0.04 M Tris base, pH 10.5, and sonicated under nitrogen for 15 min in a bath sonicator. An equal volume of 0.3 M NaCl, 0.01% sodium azide solution containing 20 μM ThT (Fluka) was added and the pH adjusted to pH 7.4. A 20-μl aliquot of Aβ_{1-40}/ThT solution was combined with 20 μl of TBSA buffer containing the peptides to be tested, in a 384-well solid white polystyrene microplate (Corning). Microplates were sealed with a plastic sheet and incubated in a PerkinElmer, model HTS 7000 Plus microplate reader at 37 °C. Plates were shaken for 1 min before fluorescent readings every 15 min using bandpass filters of 430 nm (excitation) and 485 nm (emission). In this procedure ThT fluorescence is measured throughout the assembly process, in a similar way to that previously described for monitoring light chain fibrillogenesis in real-time (25). The presence of ThT during the assembly reaction was previously found not to interfere with the formation of fibrillar Aβ (26). We have also observed using CD that 5 μM ThT does not interfere with development of the β-sheet secondary structure in Aβ (data not shown).

Circular Dichroism Spectroscopy—The incubation conditions were modified for CD studies to permit measurements down to 190 nm. Buffered solutions (5 mM Tris, pH 7.4, 100 μM EDTA) with and without either 10 μM L-KLVFFA or D-KLVFFA inhibitor peptide and 20 μM disaggregated L-Aβ_{1-40} or D-Aβ_{1-40} peptide were prepared. In addition, 10-μM solutions of D- and L-inhibitor peptides were prepared in the absence of Aβ as controls. All solutions, 100 μl, were incubated at 37 °C for 48 h in sealed 96-well solid black polystyrene microplates (Corning) as described for the ThT assay. Samples were transferred to 0.1-cm path length quartz cuvettes and CD scans were performed immediately and after 48 h using a Jasco J-715 spectropolarimeter at 37 °C between 190 and 250 nm, with a resolution of 0.1 nm and a bandwidth of 1 nm. The secondary structure content was estimated from the CD spectra using the PEPFIT program (27, 28), which has been found suitable for use with spectra from peptides such as Aβ (29).

Electron Microscopy—The solutions described above were sampled just before CD spectroscopy for preparation of platinum/carbon replicas. A 3-μl aliquot of the solution was placed onto a mica plate, air-dried, and transferred to a Balzers High-Vacuum Freeze-Etch Unit (Model 301). The specimen was shadowed with platinum under a 1.3 × 10^{-4} Pa vacuum at an angle of 30° and then coated with a carbon film. A replica was removed from the mica by flotation on deionized water and transferred to a 300-mesh transmission electron microscope grid. The grid

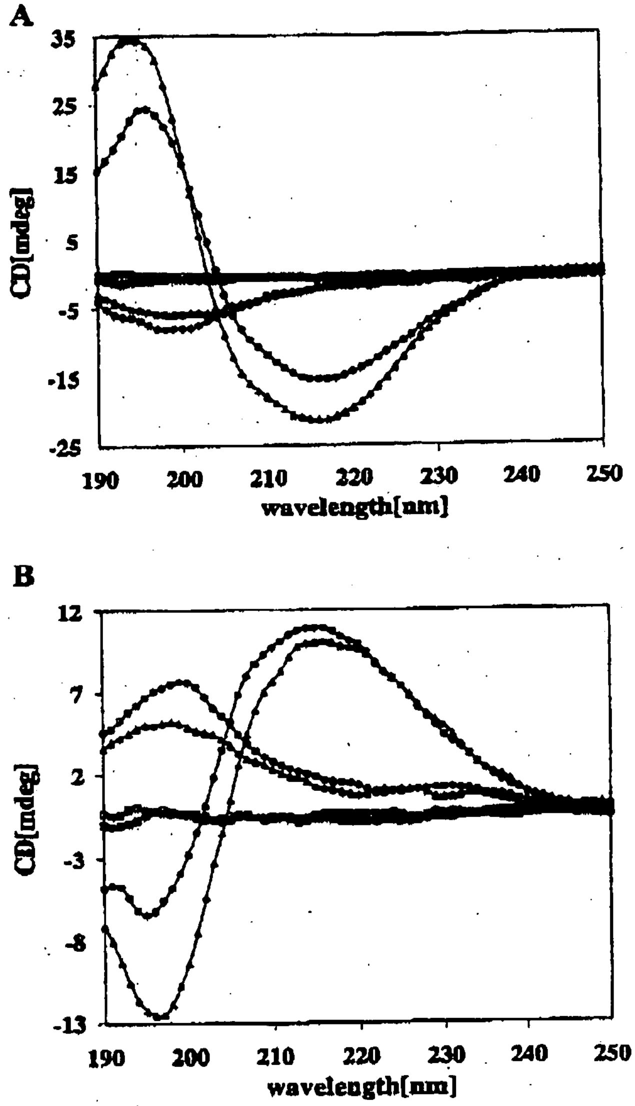

Fig. 4. The influence of KLVFFA chirality on the secondary structure transitions in Aβ_{1-40} studied by CD. *A*, L-Aβ_{1-40} was incubated at 20 μM alone or with 10 μM of either L-KLVFFA or D-KLVFFA peptide inhibitor for 48 h in Tris/EDTA buffer, pH 7.4. Peptide inhibitors were also incubated alone and their CD spectra are included in the figure to illustrate their contribution to the spectra when present with Aβ. L-Aβ before incubation (*open circles*), L-Aβ after 48 h of incubation (*filled circles*), L-Aβ with D-KLVFFA after 48 h of incubation (*open triangles*), L-Aβ with L-KLVFFA after 48 h of incubation (*closed triangles*), D-KLVFFA after 48 h of incubation (*open squares*) and L-KLVFFA after 48 h of incubation (*closed squares*). *B*, D-Aβ_{1-40} was incubated as described in *part A*. D-Aβ before incubation (*open circles*), D-Aβ after 48 h incubation (*filled circles*), D-Aβ with D-KLVFFA after 48 h of incubation (*open triangles*), D-Aβ with L-KLVFFA after 48 h of incubation (*closed triangles*), D-KLVFFA after 48 h of incubation (*open squares*) and L-KLVFFA after 48 h of incubation (*closed squares*).

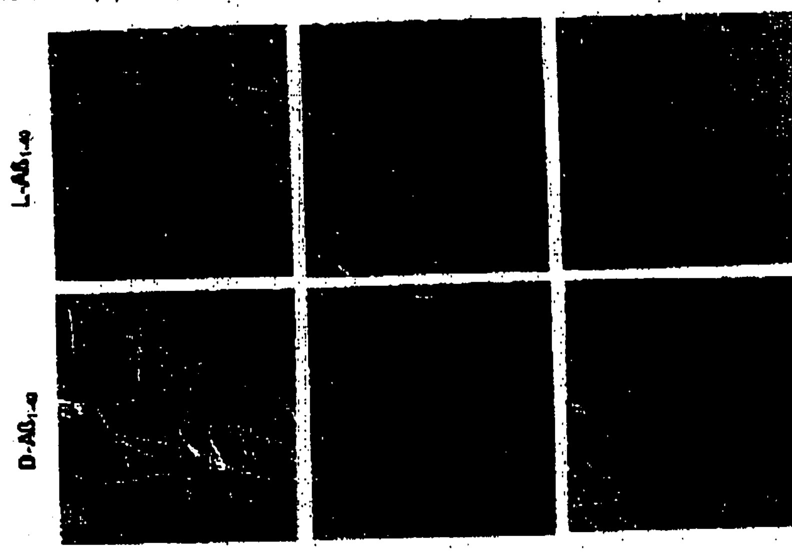

FIG. 5. The influence of KLVFFA chirality on fiber formation by L-Aβ_{1-40} and D-Aβ_{1-40} studied by EM. L-Aβ_{1-40} (*A–C*) and D-Aβ_{1-40} (*D–F*) were incubated at 20 μM with and without 20 μM L- or D-KLVFFA, as described under "Experimental Procedures" under Thioflavin T Fibrillogenesis Assay (without ThT). *A*, L-Aβ_{1-40} alone; *B*, L-Aβ_{1-40} with L-KLVFFA; *C*, L-Aβ_{1-40} with D-KLVFFA; *D*, D-Aβ_{1-40} alone; *E*, D-Aβ_{1-40} with L-KLVFFA; *F*, D-Aβ_{1-40} with D-KLVFFA. The preparations were spotted onto mica after 8 h of incubation for *A–C* and after 22 h for *D–F*. Platinum/carbon replicas were prepared and examined using a JOEL 2000FX transmission electron microscope at an original magnification of ×21,000 as described under "Experimental Procedures." The *scale bar* in *C* represents 200 nm.

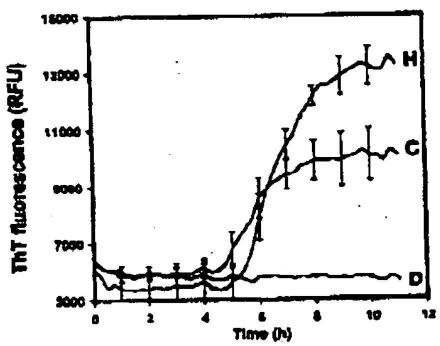

FIG. 6. D-KLVFFA inhibition of heparin-promoted Aβ_{1-40} fibrillogenesis. Aβ_{1-40} (20 μM) was incubated as described under "Experimental Procedures" for the ThT fibrillogenesis assay, either alone (*C*), with 10 μM heparin (*H*) or with both 10 μM heparin and 20 μM D-KLVFFA (*D*). Fluorescence readings of triplicate incubations taken at 15-min intervals are shown ± S.D.

was examined using a JOEL 2000 FX transmission electron microscope (JOEL) at a magnification of ×21,000.

Aβ-induced Neurotoxicity Assays—For the Aβ_{1-42}-induced neurotoxicity assay, primary cultures of rat cortical/hippocampal neurons were established from 18-day-old fetuses. After removal of meninges, cortical/hippocampal tissues were centrifuged, dissociated, and diluted to 0.8–1.0 × 10^6 cells/ml in Neurobasal B-27 medium (Invitrogen) containing 25 μM glutamate (Sigma), 0.5 mM glutamine (Invitrogen), 25 μM β-mercaptoethanol (Invitrogen), and 2% B-27 supplement (Invitrogen). The cell suspension was plated on poly-L-lysine-coated 96-well plates and incubated at 37 °C, 5% CO_2 in a humid atmosphere. On day 4 and 7 of *in vitro* culture, the medium (50 μl and 60 μl/well, respectively) was exchanged with fresh pre-warmed glutamate-free Neurobasal B-27 medium (70 μl and 80 μl/well, respectively). To evaluate the protective effect of inhibitor peptides, culture medium was completely removed from the cells and replaced by 50 μl of inhibitor peptide solution (24 or 48 μM) followed by 50 μl of Aβ_{1-42} (24 μM), both prepared in glutamate-free Neurobasal B-27 medium. The Aβ_{1-42} solution was not preincubated before addition to the cells. The cells were then incubated for a further 72-h period after which viability was assessed using the WST-1 reagent (Roche Diagnostics). Briefly, 10 μl of WST-1 reagent was added to each well and following incubation for 20–40 min the absorbance was read at 450 nm using a microplate reader (PerkinElmer, model HTS 7000 Plus). For the Aβ_{1-40}-induced neurotoxicity assay human neuroblastoma cells (SH-SY5Y, American Type Culture Collection) were cultured in a mixture (1:1) of Eagle's minimum essential medium (Sigma) and Ham's F12 medium (Cellgro), with 1% non-essential amino acids and 10% fetal bovine serum (Hyclone). The cells were trypsinized, suspended in the same medium with N2 supplement (Invitrogen), and plated at a density of 5 × 10^4 cells/well in 96-well plates. Cells were incubated to permit adherence for a 2-h period at 37 °C in 5% CO_2. The Aβ_{1-40} was preincubated for 24 h at 37 °C with or without a 4-fold molar excess of peptide inhibitor and then added to the cells. After incubation for a further 24 h, the cell viability was assessed using the MTT assay (Roche Applied Science). Briefly, 10 μl of MTT labeling reagent was added to each well for 4 h followed by addition of 100 μl of the solubilization solution. Following incubation for 16 h at 37 °C, the absorbance was read at 560 nm using a microplate reader. For both Aβ_{1-42} and Aβ_{1-40}, the percent cell viability was calculated as being equal to [(A_{450} sample − A_{450} blank)/(A_{450} control − A_{450} blank)] × 100.

RESULTS

D-Enantiomers of Inhibitor Peptides More Potently Inhibit Aβ Assembly than L-Enantiomers—To evaluate the effect of chirality on the potency of peptide inhibitors of Aβ assembly, two enantiomeric pairs of inhibitor peptides were compared. One of these peptides, KLVFFA, corresponds to the primary sequence of Aβ_{16-21}, an Aβ self-recognition motif (16). A second peptide, with an additional lysine at the N terminus, KKLVFFA, was also tested, as it was previously demonstrated to bind specifically to peptides containing the Aβ_{16-21} KLVFF motif (17). The relative ability of these peptides to inhibit Aβ assembly was assessed using the amyloid specific dye ThT, which increases in fluorescence as Aβ peptides assemble into fibrillar structures (26). Both KLVFFA and KKLVFFA inhibited about 16-fold more potently when in the D-form compared with the L-form. L-KLVFFA exhibited antifibrillogenic activity at 400 μM (Fig. 1*A*), which was similar to that of D-KLVFFA at 25 μM (Fig. 1*C*). L-KKLVFFA at 400 μM (Fig. 1*D*) inhibited slightly less effectively than did D-KKLVFFA at 25 μM (Fig. 1*F*). Three additional related peptide pairs, KIVFFA, KFVFFA, and KVVFFA, were produced by substitutions at the leucine, a residue previously shown to be critical for binding (16). The results of testing these at 20 μM (KIVFFA and KFVFFA) or at 100 μM (KVVFFA) are presented in Fig. 2. After incubation for 10 h, the D-forms were found to be more inhibitory in each case. We observed that while replacement of the leucine by isoleucine or phenylalanine did not greatly change the inhibitor activity compared with the parent peptide D-KLVFFA, replacement by valine did reduce the inhibition. Replacement by alanine (data not shown) caused a near complete loss of inhibitor activity, consistent with the much reduced binding of ^{125}I-Aβ_{1-40} to solid phase bound D-KAVFF compared with L-KLVFF reported previously (16). It is concluded that inhibitor peptides interacting with the KLVFF motif, inhibit Aβ fibrillogenesis more potently when in the D-form.

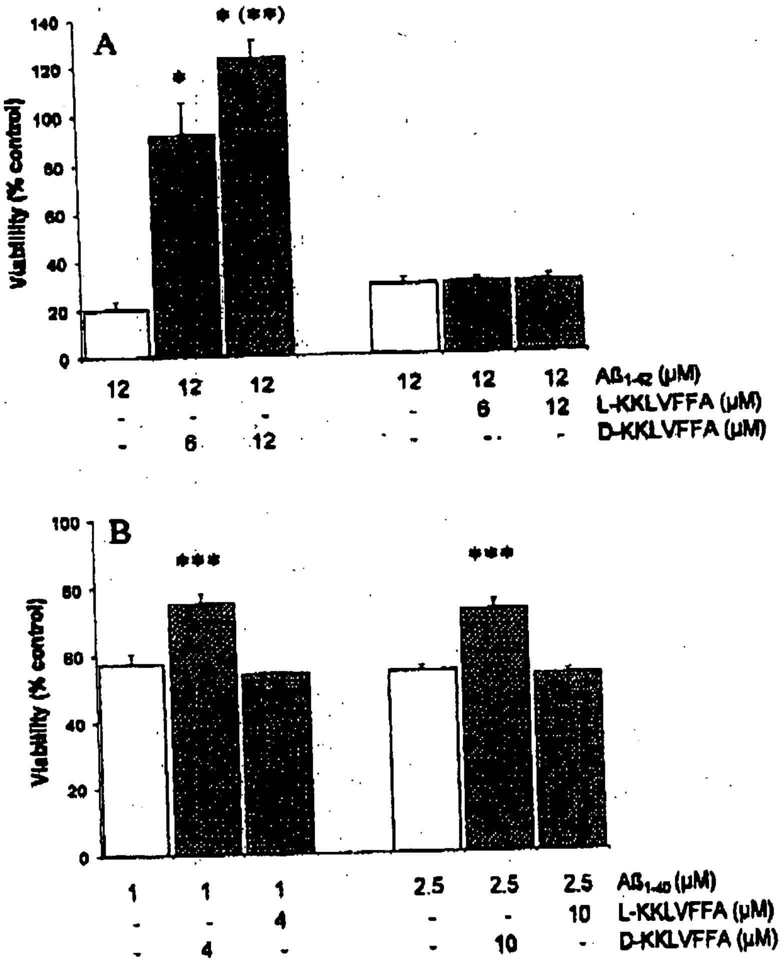

FIG. 7. **Effect of D- and L-inhibitor peptides on Aβ-induced toxicity in cell culture.** *A*, freshly prepared $A\beta_{1-42}$ (12 μM), alone or with 6 or 12 μM of either D- or L-KKLVFFA, was added to primary cultures of rat neurons and incubated for 3 days. Cell viability was determined using WST-1, (n = 6). *B*, $A\beta_{1-40}$ (1 or 2.5 μM), preincubated 24 h alone or with 4 or 10 μM of either D- or L-KKLVFFA, was added to cultures of SH-SY5Y human neuroblastoma cells and incubated a further 24 h. Cell viability was determined using MTT, (n = 3). *, significantly different, $p < 0.001$, compared with $A\beta_{1-42}$ without inhibitors; **, significantly different, $p < 0.001$, compared with $A\beta_{1-42}$ with D-KKLVFFA at 6 μM; ***, significantly different, $p < 0.001$, compared with $A\beta_{1-40}$ without inhibitors and compared with $A\beta_{1-40}$ with an equal concentration of L-KKLVFFA. The statistical analysis consisted of a one-way analysis of variance test followed by a Tukey test (SigmaStat, Version 2.03, SPSS Inc.).

Enantiomeric Aβ (D-$A\beta_{1-40}$) Exhibits Reciprocal Chiral Inhibitor Specificity—Enantiomeric proteins exhibit reciprocal chiral specificity with respect to their biochemical interactions (23). Aβ synthesized entirely of D-amino acids (D-Aβ) was used to re-assess the effect of L- and D-forms of KLVFFA on Aβ assembly by ThT. As shown in Fig. 3, in the case of D-Aβ assembly, the L-enantiomer of KLVFFA was the more potent inhibitor.

D-Peptide Inhibitors Most Effectively Reduce the Transition of $A\beta_{1-40}$ to β-Sheet Secondary Structure—Aβ transitions to a β-sheet-rich secondary structure during assembly into amyloid fibrils. We used CD to assess whether the inhibitor peptides could prevent Aβ from adopting a β-sheet conformation, and to determine which enantiomer was the most effective. L-$A\beta_{1-40}$ before incubation exhibited a CD spectra, which when deconvoluted (27) corresponded to a secondary structure of 60% random coil and 40% β-sheet (C of c = 0.96, Fig. 4*A*). L-$A\beta_{1-40}$ after incubation in the absence of peptide inhibitors, transitioned to a secondary structure 60% β-sheet and 40% α-helix (C of c = 0.98; Fig. 4*A*). The presence of 10 μM D-KLVFFA during the L-Aβ incubation prevented this random coil to β-sheet-rich secondary structure transition, maintaining Aβ close to its original conformation, 56% random coil and 44% β-sheet (C of c = 0.92; Fig. 4*A*). The addition of 10 μM L-KLVFFA to the incubation of $A\beta_{1-40}$ permitted similar structural transitions to those observed when Aβ was incubated alone, which corresponded to 40% β-sheet and 60% α-helix (C of c = 0.98; Fig. 4*A*). Identical experiments performed using D-$A\beta_{1-40}$ are presented in Fig. 4*B*. In this case the CD spectra are mirror images of those produced by L-$A\beta_{1-40}$. The presence of D-KLVFFA had little effect on the CD spectra of D-Aβ after a 48-h incubation. In contrast, the addition of L-KLVFFA maintained the D-Aβ in a structure similar to that observed before incubation. Comparable results were obtained when similar experiments were performed in TBSA buffer, which contains physiological levels of salt (data not shown).

D-Peptide Inhibitors More Potently Reduce Fiber Formation by L-$A\beta_{1-40}$—EM was used to determine the effect of the enantiomeric inhibitor peptides on $A\beta_{1-40}$ fiber formation. L-$A\beta_{1-40}$ (20 μM) incubated alone (Fig. 5*A*) or in the presence of 20 μM L-KLVFFA (Fig. 5*B*) formed large fibrillar structures with the characteristic morphology of Aβ amyloid. However, when 20 μM D-KLVFFA was present during the incubation, very few or no fibrillar structures were observed (Fig. 5*C*). Conversely, when 20 μM D-$A\beta_{1-40}$ was incubated either alone (Fig. 5*D*) or in the presence of 20 μM D-KLVFFA (Fig. 5*F*), numerous densely packed fibrils were observed. In the presence of 20 μM L-KLVFFA, D-$A\beta_{1-40}$ did not form fibrils (Fig. 5*E*).

The D-KLVFFA Inhibitor Peptide Effectively Blocks Aβ Assembly Facilitated by Pathological Chaperones—The effect of the D-KLVFFA inhibitory peptide was tested in an Aβ fibrillogenesis assay in the presence of heparin. This glycosaminogly-

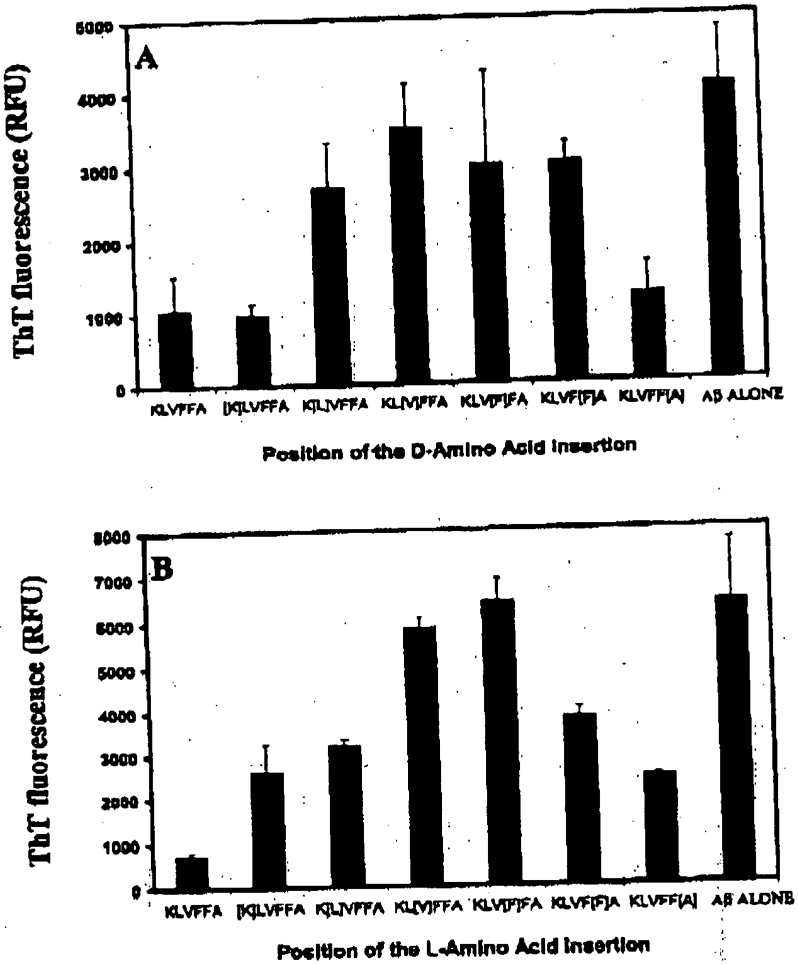

Fig. 8. Effect on inhibitor peptide of switching the chirality of individual amino acid residues. *A*, L-KLVFFA and its six diastereoisomers prepared by substitution of single D-amino acids at each residue position were tested for antifibrillogenic activity at 200 μM as described under "Experimental Procedures" using the ThT fibrillogenesis assay; *B*, D-KLVFFA and its six diastereoisomers prepared by substitution of single L-amino acids at each residue, tested at 200 μM. The average of triplicate incubations is shown ± S.D.

can acts as a natural pathological chaperone, facilitating the formation of amyloid fibrils *in vitro* (30) and is believed to play a role in Aβ fibrillogenesis *in vivo* (13). In our Aβ assembly assay, heparin increased the ThT fluorescence by 1.8-fold. When the D-KLVFFA peptide was added to the incubations containing both Aβ and heparin, no increase in ThT fluorescence was observed, indicating a complete inhibition of chaperone catalyzed Aβ assembly (Fig. 6).

D-Inhibitor Peptides Selectively Protect Against L-$A\beta_{1-42}$ *and* L$A\beta_{1-40}$ *Toxicity in Cell Culture*—$A\beta_{1-42}$ at 12 μM reduced the viability of cultured rat primary cortical neuronal cells to 20 and 29% of that observed in control cells incubated without $A\beta_{1-42}$ (Fig. 7*A*). In the presence of 6 or 12 μM D-KKLVFFA, the toxicity of 12 μM $A\beta_{1-42}$ was significantly attenuated ($p <$ 0.001). The resulting cell viabilities were 91.9% and 123.1% of control cells respectively, with the effect of 12 μM peptide being significantly greater ($p < 0.001$) than that of 6 μM peptide. In the presence of 6 or 12 μM, of L-KKLVFFA the toxicity of 12 μM $A\beta_{1-42}$ persisted, and the viability remained similar to that seen without addition of the peptide inhibitors, 29.5% and 29.4% of control cells, respectively.

$A\beta_{1-40}$ at 1 μM reduced viability of SH-SY5Y human neuroblastoma cells to 57.3% of control levels (Fig. 7*B*). The cell viability in the presence of $A\beta_{1-40}$ was significantly improved to 75% of control levels ($p < 0.001$) by the addition of 4 μM D-KKLVFFA, but was not significantly changed, 54%, after the addition of 4 μM L-KKLVFFA. $A\beta_{1-40}$ at 2.5 μM reduced viability to 54.3% of control. This toxicity was prevented by the addition of 10 μM D-KKLVFFA, which resulted in a viability of 75% of control cells ($p < 0.001$). This was not seen with the addition of 10 μM L-KKLVFFA, which resulted in a viability of 52.7%, not different from that seen with 2.5 μM Aβ alone.

Full Potency of D-Peptides Requires All D-Amino Acids—We investigated the antifibrillogenic activity of L-KLVFFA after the systematic replacement of each L-residue by its corresponding D-amino acid (Fig. 8*A*). Each such chiral switch of a single residue resulted in a loss of antifibrillogenic activity except in the case of the N-terminal lysine residue. The greatest losses in activity were observed when the L-amino acids from the central region of the peptide (residues 17 to 20) were substituted. Similarly, all single D- to L-substitutions in D-KLVFFA decreased the potency of the D-enantiomer (Fig. 8*B*). Again, the greatest loss of potency occurred when the chiral inversion was made at the center of the peptide.

D-Peptides Containing the $A\beta_{16-20}$ *Motif Inhibit Aβ Assembly by Interacting at the Complementary Region of Aβ*—Self-recognition of the $A\beta_{16-20}$ KLVFF motif is believed to take place with the L-enantiomer inhibitor peptides described thus far (17, 19). To establish whether this is also the case with the D-enantiomers of peptide inhibitors, a series of overlapping 6 and 7 D-peptides spanning the entire length of Aβ were synthesized and evaluated using the ThT fibrillogenesis assay (Fig. 9). The only peptides found to exhibit inhibitory activity, D-$A\beta_{16-22}$ and D-$A\beta_{15-20}$, contained the entire $A\beta_{16-20}$ sequence.

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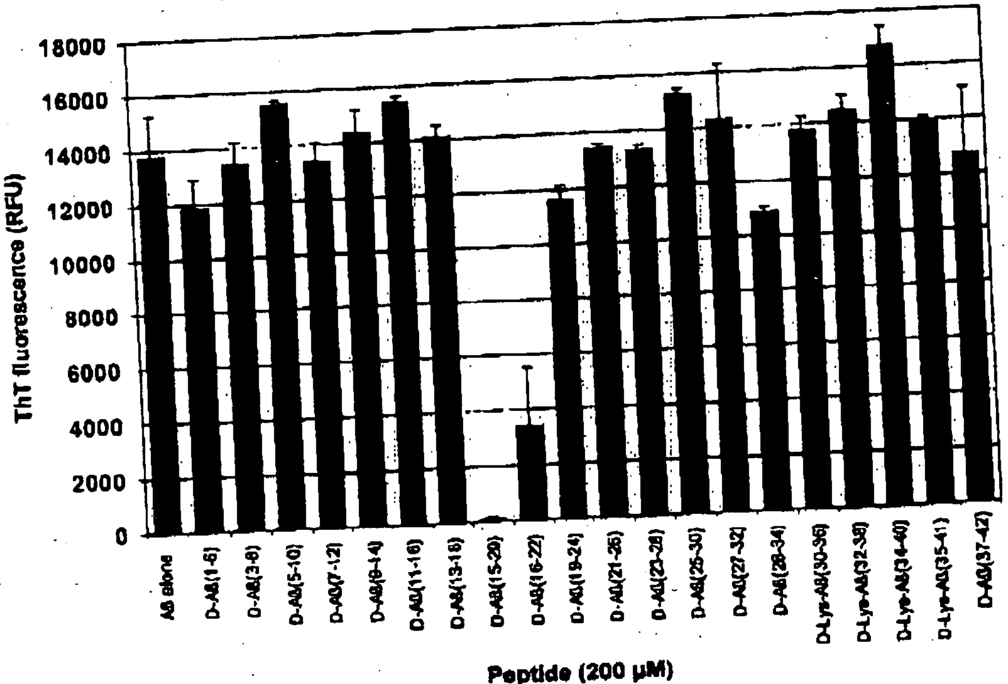

FIG. 9. **Inhibitory activity of D-peptides corresponding to other regions of Aβ.** A series of twenty 6- and 7-mer D-peptides were synthesized, corresponding to overlapping sections of Aβ. $A\beta_{1-40}$ at 20 μM and individual D-peptides at 200 μM were incubated as described in the "Experimental Procedures" under the ThT fibrillogenesis assay. The average of triplicate incubations is shown ± S.D.

DISCUSSION

Inhibition of Aβ Assembly by Peptides Containing the KLVFF Aβ-Recognition Motif Is Heterochirally Selective—Protein/protein interactions can be highly stereospecific. For example, the HIV-1 protease cleaves only the naturally occurring L-form of its substrate peptide, thus exhibiting a homochiral specificity. A synthetically prepared D-enantiomer of this HIV-1 protease maintains the homochiral specificity characteristic of the native enzyme, by acting only on the D-form of its substrate (28). Analogously, the ribonuclease S-protein specifically binds the L-form, but not the D-form of the S-peptide to form an active complex (31). In other cases, protein/protein interactions exhibit no chiral preference. For example, a synthetic HIV-1 GP41 fusion peptide inhibits HIV-1 envelope glycoprotein mediated-cell fusion both in the D- and L-form indicating that the peptide's chirality is not critical for peptide/peptide interactions (32). Similarly the co-chaperone DnaJ-bound D-peptides and L-peptides with comparable affinities (33).

We show here that inhibitor peptides containing the entire KLVFF Aβ-recognition motif inhibited Aβ assembly with a heterochiral type of stereospecificity. D-Enantiomers of these inhibitor peptides preferentially inhibited the assembly of the natural L-enantiomer of Aβ. Conversely, L-enantiomers of these inhibitor peptides preferentially inhibited the assembly of the synthetic D-enantiomer of Aβ. We have demonstrated this unusual heterochiral specificity in Aβ assembly assays using the amyloid specific dye ThT. We have also observed this specificity in the inhibition of the structural transition of Aβ to β-sheet employing CD and in the inhibition of Aβ fiber formation employing EM. The hetereochiral specificity of the inhibitor peptides was also evident in their biological activity, as the D-peptides more effectively blocked Aβ-induced neurotoxicity in cultured neurons.

We have considered alternative explanations for our observations. For instance the possible selective contamination of L-inhibitor peptides by aggregation promoting metal ions such as copper (34) would cause an apparent reduction in the potency of this enantiomer in our assays. However such a possibility was excluded based on our results showing that the L-peptides had greater potency than the D-peptides, when inhibiting the assembly of D-$A\beta_{1-40}$. This demonstrated that the lower potency of the L-peptides with L-$A\beta_{1-40}$, is an inherent characteristic of the interaction, and not attributable to a contaminant.

We have considered the possibility that when an inhibitor peptide of opposite chirality is combined with Aβ the resulting solution fails to develop a β-sheet-type spectra because of spectral subtraction rather than because of inhibition of a change in Aβ structure. We do not believe this to be the case here, because we observe a spectrum for the solution of Aβ and inhibitor which exhibits a negative minimum at around 197 nm characteristic of high random coil content. If in the mixture of Aβ and inhibitor of opposite chirality both had converted to a high β-sheet secondary structure, the resulting CD spectra would instead have cancelled each other out, at all wavelengths, producing a flat spectrum at zero mdeg. If the spectral cancellation were not complete, we would have expected some residual positive or negative signal at the 218 nm, characteristic of β-sheet. The results we observe are more consistent with the peptide inhibitors causing a true inhibition of the conversion of Aβ to a high β-structure form.

The $A\beta_{16-20}$ Region Is the Site of D-Peptide Inhibitor Interaction with Aβ—A previous study reported that peptides containing the KLVFF Aβ-recognition motif interact with Aβ through the homologous primary sequence, in a homochiral-specific manner, when studied using a direct binding assay (17). In view of this report we questioned whether the D-peptide inhibitors function by interacting with Aβ through this site. To establish whether this indeed was the case, we prepared and tested a series of 20 overlapping 6- and 7-mer D-peptides cor-

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responding to the sequence of Aβ beginning at the N-terminus. From the ThT assay results it was clear that only peptides incorporating the KLVFF motif, namely $A\beta_{15-20}$ and $A\beta_{16-22}$, possessed antifibrillogenic activity. This suggests that the D-peptide inhibitors do indeed interact with Aβ via the KLVFF motif, just as do the L-peptides. It remains unclear why a homochiral stereospecificity was observed for the direct binding of Aβ to immobilized peptides containing the KLVFF motif (17) while a heterochiral stereospecificity was observed for the antifibrillogenic activity of the inhibitor peptides described in the present report. The replacement of amino acids by their enantiomers causes a local change of secondary structure (35) which we observed resulted in a loss of inhibitor potency (Fig. 8). It is possible that the solid phase attachment of the D-peptide (17) in a similar manner restricted its conformation to ones that do not interact with Aβ.

D-KLVFFA Inhibits Aβ Assembly Promoted by a Pathological Chaperone—Certain non-Aβ components that are associated with AD plaques promote Aβ assembly and are therefore referred to as "pathological chaperones" (36, 37). Glycosaminoglycans are important examples of such pathological chaperones that induces both β-sheet structure and amyloid fibrils in Aβ (30). For an inhibitor to function effectively *in vivo*, inhibition of chaperone-mediated fibrillogenesis would be a requirement. D-KLVFFA, when added to incubations promoted by heparin, completely inhibited fibrillogenesis, clearly demonstrating that not only does this peptide inhibit Aβ assembly, it is also effective when a chaperone plays a catalytic role in the process. Further testing would be required to determine whether this inhibitor peptide is active with other pathological chaperones as well.

D-Enantiomer Inhibitor Peptides are More Potent Than Are L-Forms at Countering Aβ Neurotoxicity—We found that the D-enantiomer of KKLVFFA was more potent than the L-form in preventing the neurotoxicity of Aβ *in vitro*. This neuroprotective activity is consistent with the prevention of Aβ assembly by this inhibitor peptide. As the toxicity of Aβ has been shown to require some type of supramolecular assembly, the ability of D-peptides to prevent such intermolecular interactions by Aβ understandably leads to neuroprotection in cell culture experiments. This effect was demonstrable with $A\beta_{1-40}$ and $A\beta_{1-42}$, the two forms of Aβ prominently found in AD. The greater neuroprotective effect of the D-enantiomer was evident both in primary culture of rat neurons and in cultures of neuroblastomal cells. Although the L-enantiomer failed to exhibit neuroprotective effects under the conditions tested here, a higher concentration could have proven effective. This is suggested by the ability of higher concentrations of the L-inhibitors to prevent the ThT fluorescence of L-Aβ. Indeed, previous studies have reported modulation of Aβ toxicity by L-enantiomeric derivatives containing the KLVFF motif (38). Showing that D-peptides are more potent than their L-counterparts at reducing Aβ toxicity indicates that they could constitute useful drug leads for therapeutic interaction in AD.

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that time or because they were not part of the prescribed minimum documentation. The national laws on patentability, that is to say, on novelty, inventive step and industrial applicability or the equivalent thereto will usually differ to a greater or lesser extent from the corresponding criteria for the international preliminary examination (Article 27(5) PCT). For this reason, the international preliminary examination report is not binding on elected Offices (Article 33(1) and (5) PCT).

It is the practice of many designated Offices to require the applicant in a national application to supply details of prosecution of corresponding applications in other countries, especially when priority is claimed. In the case of an international application, this practice is restricted in that one elected Office cannot require the applicant to supply particulars of prosecution of the same international application before any other elected Office (Article 42 PCT).

One of the matters which may be raised in the national phase is whether the claims are directed to more than one invention. This is discussed at paragraph 33.35.

The grant or refusal of a patent or other kind ofprotection on an international application is the prerogative of the designated Offices in accordance with their national laws (Articles 11(3) and 27(5) PCT). The decisions on such matters by the designated Offices are open to appeal to higher authorities in exactly the same way as decisions on national applications are open to appeal (as to which see para. 33.48).

33.35 Unity of invention in the national phase

One of the matters which may be considered by designated Offices in the national phase is whether the claims are directed to more than one invention. If a designated or elected Office considers that a finding by the International Searching or Preliminary Examining Authority that there is a lack of unity of invention is justified and the applicant did not pay the additional search or examination fee in response to the Searching or Examining Authority's invitation, the unsearched or unexamined claims may be considered withdrawn unless the applicant pays a special fee to the designated or elected Office (Articles 17(3)(b) and 34(3)(c) PCT). This could presumably, national law permitting, give the applicant the opportunity to pursue the unsearched or unexamined claims before the designated Office, instead of the searched or examined claims or to have a national search or examination conducted against the claims unsearched or unexamined in the international phase prior to deciding whether to file a divisional application at the designated Office.

The PCT Contracting States have agreed to follow the practice under the Patent Cooperation Treaty on unity of invention (as to which see para. 23.9). Therefore, a designated Office ought not to raise an objection as to a lack of unity when the International Searching and/or Preliminary Examining Authority has found that the claims comply with the requirement for unity of

invention (Rule 13 PCT). When the issue of unity of invention has been raised in the international phase and a protest against an invitation to pay additional search or examination fees has been upheld (paras 29.13 and 34.34 respectively), the applicant can ask the International Searching or Preliminary Examining Authority, as the case may be, for copies of the decision on the protest to be supplied to the designated Offices. A translation of such decision should accompany any prescribed translation of the international application itself (paras 33.7 and 33.16).

33.36 Non-prejudicial disclosures

Many national laws contain provisions for excluding certain disclosures of an invention before the filing date or before the priority date of a national patent application from consideration when deciding whether that invention as claimed in the national application meets the criteria for patentability, having regard to the prior art. Such disclosures are usually called "non-prejudicial disclosures" or "exceptions to lack of novelty". Depending on the national law, a non-prejudicial disclosure might be a disclosure without the consent of the applicant (a wrongful disclosure or an "evident abuse"), a disclosure of the invention at an exhibition, especially an international exhibition within the terms of the 1928 Convention on International Exhibitions, as amended, or a disclosure of the invention by or with the consent of the inventor or the applicant within a grace period. Such non-prejudicial disclosures are mentioned in the 1963 Strasbourg Convention on Unification, Articles 4(4) and 12(1)(b) (Appendix VIII of this Handbook). Provisions concerning disclosures at official or officially recognized international exhibitions are also contained in the Paris Convention, Article 11 (Appendix VII). Some national laws require that particulars of the circumstances of a disclosure be filed with the patent application in order that the disclosure shall be non-prejudicial.

The Patent Cooperation Treaty itself does not contain any provisions as to non-prejudicial disclosures but Rule 4.17(v) PCT provides a mechanism for furnishing particulars of non-prejudicial disclosures or exceptions to lack of novelty in the international phase in order to comply with requirements of national laws applicable in designated States. If a declaration as to non-prejudicial disclosures or exceptions to lack of novelty, using the standardized wording prescribed in Section 215 of the PCT Administrative Instructions, is appropriate for the designated Offices identified therein, such a declaration may have been included in the request at the time of filing, as described in paragraph 21.10A, in order that the particulars as to the non-prejudicial disclosures shall have been furnished at the time of filing of the international application. Alternatively, such a standardized declaration as to non-prejudicial disclosures, etc., may have been furnished later in the international phase, as described in paragraph 27.12B, although the later furnishing of particulars as to a non-prejudicial disclosure may not meet the requirements of the national laws applicable at some designated Offices. There is also the possibility of particulars of